

Molecular Systematics of the *Drosophila hydei* Subgroup as Inferred from Mitochondrial DNA Sequences

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Abstract. The phylogeny of the *Drosophila hydei* subgroup, which is a member of the *D. repleta* species group, was inferred from 1,515 base pairs of mitochondrial DNA sequence of the cytochrome oxidase subunits I, II, and III. Four of the seven species in the subgroup were examined, which are placed into two taxonomic complexes: the *D. bifurca* complex (*D. bifurca* and *D. nigrohydei*) and the *D. hydei* complex (*D. hydei* and *D. eohydei*). Both complexes appear to be monophyletic, although the *D. bifurca* complex is only weakly supported. The evolution of chromosomal change, interspecific crossability, sperm gigantism, and divergence times of the subgroup is discussed in a phylogenetic context.

Key words: DNA sequence variation — Maximum likelihood — Γ -distributed rates model — Cytochrome oxidase — Molecular clock

Introduction

The *Drosophila hydei* subgroup is one of five subgroups in the *D. repleta* species group. The subgroup was initially erected by Wharton (1944) based on morphological and genetic similarities, although no morphological characters were presented and the genetic similarities were

not diagnostic. Wheeler (1949) was the first to diagnose the subgroups within the *D. repleta* group based on morphological criteria, including the *D. hydei* subgroup. Wasserman (1962) better defined the subgroup morphologically and cytologically, and Villela (1983) updated the diagnoses to give the current definition of the *D. hydei* subgroup. The primary morphological characters used to define the subgroup are the number of coils in the ventral receptacles and the number of coils in the testes. In each of these organs, the number of coils correlates with organ length. Organ length, in turn, is highly correlated with sperm length (Hihara and Kurokawa 1987; Joly and Bressac 1994), as the testes and seminal receptacles must be longer than the sperm they manufacture and store, respectively. Sperm length has been evolutionarily labile in the genus *Drosophila*, with very long sperm having independently evolved numerous times (Pitnick et al. 1995a).

The subgroup currently consists of seven species (accepting the synonymy of *D. hydeoides* into *D. nigrohydei*), all of which are described in detail by Wasserman (1962) and Villela (1983). Figure 1 presents the previous phylogeny for the subgroup, which is based on Wasserman (1982, 1992). The subgroup is divided into two groups: the *D. hydei* complex (*D. hydei*, *D. eohydei*, *D. neohydei*) and *D. bifurca* complex (*D. bifurca*, *D. nigrohydei*, *D. novemaristata*, *D. guayllabambae*). But this taxonomic division is not based on derived characters which are necessary for phylogenetic reconstruction, so the evolutionary existence of these groups cannot be ascertained. As is apparent from Fig. 1, phylogenetic rela-

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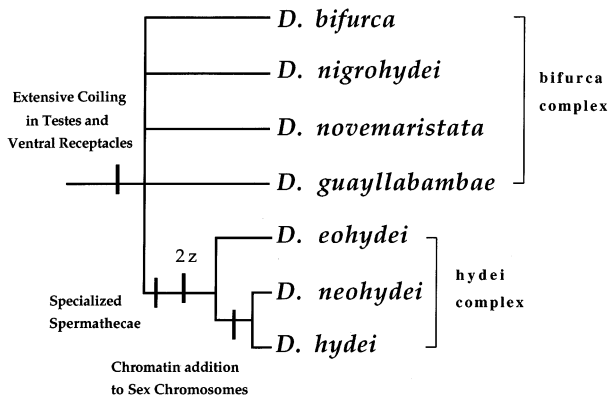


Fig. 1. Phylogeny of the *D. hydei* subgroup based on the morphological and chromosomal characters which have been used to infer the traditional classification of the subgroup (Wasserman 1982, 1992). The inversion 2z is described in the text.

tionships among the species still remain essentially unresolved using traditional character sets.

While cytological information has been useful in determining the relationships among species in the *D. repleta* group, the *D. hydei* subgroup has experienced very few chromosomal rearrangements. Karyotypically it appears to possess the ancestral inversion configuration of the *D. repleta* species group, designated Primitive I by Wasserman (1992). Although the subgroup has 14 intra-specific inversions reported (Wasserman 1992), there is only one fixed inversion (2z), which is shared among the subgroup species (Wasserman 1954, 1982). The chromosome 2 inversion z unites *D. hydei*, *D. eohydei*, and *D. neohydei* in the *D. hydei* complex.

Within the *D. hydei* complex, Wasserman (1992) suggests ancestral-descendent relationships based on the progressive addition of heterochromatin to the sex chromosomes. His evolutionary scheme proposes that *D. neohydei* possesses the ancestral condition of an acrocentric X and small acrocentric Y karyotype which progressively adds heterochromatin to evolve into a medium submetacentric X and a large acrocentric Y in *D. eohydei*, and then to a large metacentric X and submetacentric Y in *D. hydei*. While this may be justified, an outgroup assessment evaluating the *D. bifurca* complex indicates that the sex chromosomes in that complex have also experienced heterochromatin addition/deletion. Therefore, trying to determine the direction of evolution in this character could be problematical, because the subgroup exhibits such variation for this character. The situation for the *D. bifurca* complex is even more ambiguous, due to a lack of useful characters. Accordingly, no phylogenetic relationships have been proposed for species in the *D. bifurca* complex.

Resolving phylogenetic relationships among the *D. hydei* subgroup species is a matter of importance to biologists concerned with the maintenance of anisogamy and the evolution of reproductive strategies. The reproductive biology of these species is unusual, being char-

acterized by males exhibiting the female-like strategy of producing relatively few, high-investment gametes. In fact, *D. bifurca* and *D. hydei* have the two longest known spermatozoa of the animal kingdom (Pitnick et al. 1995b; Pitnick and Markow 1994). Production of these giant sperm is costly; in *D. bifurca*, for example, the 67-mm-long testes comprise nearly 11% of males' total dry body mass (Pitnick 1996). Time required to grow these large testes appears to be causally responsible for the unusually delayed rates of male sexual maturation in these species (Pitnick et al. 1995a). Moreover, in *D. hydei* the differential ability of males to invest in sperm production contributes to body-size-related variation in male reproductive success (Pitnick and Markow 1994).

Detailed knowledge of the evolutionary history of these species is fundamental for discerning direction and rates of evolutionary modification in characters such as sperm length, the respective roles of phylogenetic constraint and ecological adaptation in shaping these characters, as well as patterns of correlated evolution between sperm length and other reproductive and life history traits. We therefore examined the phylogenetic relationships among the four available species (*D. hydei*, *D. eohydei*, *D. bifurca*, and *D. nigrohydei*) in the *D. hydei* subgroup by sequencing 1,515 base pairs of the mitochondrial DNA (mtDNA)-encoded cytochrome oxidase subunits I, II, and III (COI-III).

Materials and Methods

The DNA sequences of the mitochondrial cytochrome oxidase I, II, and III subunits from four species of the *D. hydei* subgroup were obtained by the direct sequencing of polymerase chain reaction (PCR) products. The corresponding mtDNA sequences from *D. yakuba* and *D. melanogaster*, which were used as outgroups in this study, came from Clary and Wolstenholme (1985) and de Bruijn (1983), respectively.

The oligonucleotides that were used for PCR amplification and sequencing are listed according to the system established by Simon et al. (1994). The amplification primers for COI (C1-J-1751 and C1-N-2191) were initially developed in R. Harrison's laboratory at Cornell University, but these have been made specific to *Drosophila*. Most of the primers for COII (TL2-J-3037 and TK-N-3785) appeared in Liu and Beckenbach (1992) and Beckenbach et al. (1993), although some have been modified from the original compilation. The amplification primers for COIII (C3-J-5014 and C3-N-5460) were designed by C. Simon and C. Orego and appeared in Simon et al. (1991). Most of the internal sequencing primers were designed independently and can be found in Spicer (1995).

The strains that were used, and their corresponding National Drosophila Species Resource Center stock numbers, where applicable, are as follows: *D. hydei*, Tempe, Arizona, 1989, collected by Teri A. Markow; *D. eohydei* (15085-1631.0), Santa Marta Mountains, Colombia; *D. bifurca* (15085-1621.0), Metztlán, Hidalgo, Mexico; *D. nigrohydei* (15085-1661.1), Portal, Arizona. The other three species in the *D. hydei* subgroup were not included in this study due to our inability to obtain specimens. Both *D. novemaristata* and *D. guayllabambae* are known only from their species descriptions, and while *D. neohydei* has been the subject of numerous previous studies, it is apparently no longer maintained in laboratory culture.

Total genomic DNA was isolated by grinding one to five male flies with a Teflon grinding implement. This was performed in a 1.5-ml tube

containing 500 μ l of grinding buffer (0.1 M EDTA, 100 mM Tris pH 8.0, 1% SDS, 0.2 M NaCl). The homogenate was incubated overnight at 65°C and then extracted with equilibrated phenol several times until the supernatant was not cloudy or discolored. The supernatant was then extracted two times with chloroform, then with cold 100% ethanol, and finally several times with 70% ethanol at room temperature. The DNA was dried and resuspended in 200 μ l of d.d. water.

The conditions of the PCR (Mullis et al. 1987; Saiki et al. 1988) were varied depending on the genes being amplified. The double-stranded amplifications reaction volumes were usually 50- μ l solutions. Generally, the 5 \times buffer (300 mM Tris-HCl, 75 mM (NH₄)₂SO₄, pH 8.5) was used, along with 10 mM of dNTPs and 10 μ M of the primers. Both the Mg⁺⁺ concentration and pH were adjusted depending on the template. These were varied from a concentration of 7.5–17.5 mM of MgCl₂ and a pH 8.5–9.5 for the buffer. Between 30 and 35 cycles were used for the amplifications. The denaturing step was set at 94°C for 40 s and the extension step was at 72°C for 1 min. The annealing step varied according to the primers that were being amplified. Usually, this ranged from 48 to 54°C for 2 min. The double-stranded templates were purified using the Pharmacia Biotech MicroSpin S-300 HR columns according to the protocol supplied by the manufacturer.

All direct DNA sequencing of the double-stranded PCR products was performed using the USB Sequenase Kit, but not according to the manufacturer's guidelines. Instead, a modified protocol was followed (Casanova et al. 1990; Liu and Beckenbach 1992). The denaturing step consisted of boiling the template, reaction buffer, and primer for 5 min and then placing this into either a liquid nitrogen or a dry ice/ethanol bath. The labeling reaction mixture was added while the sample was still frozen. The reaction mixture was microfuged for 30 s and then added to the extension mix. This was incubated at 37–42°C for 5 min, then terminated.

A variety of techniques were used to infer the phylogenetic relationships among the taxa. Parsimony analyses were performed using PAUP*Star (Swofford 1996). These were accomplished with the branch-and-bound algorithm (Hendy and Penny 1982) using both unordered changes and step-matrices to differentially weight transitions and transversions. When several equally parsimonious trees were found, strict consensus trees (Rohlf 1982) were produced to summarize these data. In order to assess some confidence limits concerning the branching pattern, a bootstrap analysis was performed (Felsenstein 1985). A total of 300 replications were performed using the branch-and-bound algorithm. The result is presented as a majority-rule consensus tree (Margush and McMorris 1981), which shows the most frequently occurring branching orders. In addition, to evaluate some alternative less-parsimonious arrangements, tree manipulations were accomplished by using the program MacClade (Maddison and Maddison 1992). Distance analyses were also executed with the program PAUP*Star (Swofford 1996). These consisted of simple percentage calculations (*p*-distance), Jukes-Cantor distance (Jukes and Cantor 1969), Kimura two-parameter model (Kimura 1980), Tamura-Nei distance (Hasegawa et al. 1985; Tamura and Nei 1993), and the Log Det/Paralinear procedure (Lockhart et al. 1994; Lake 1994). These distance measures were then clustered by using UPGMA (Sokal and Sneath 1963) and the minimum evolution method (Saitou and Imanishi 1989). A bootstrap confidence level was determined for each tree. In addition, among-site rate variation was incorporated into the analyses by using the Kimura two-parameter gamma (Jin and Nei 1990) and Tamura-Nei gamma (Tamura and Nei 1993) corrected distances. Finally, maximum likelihood was used to estimate parameters and evaluate the molecular clock hypothesis, and these analyses were also conducted using PAUP*Star (Swofford 1996).

Results

The sequenced regions of the mitochondrial cytochrome oxidase gene encompass a 408-base-pair (bp) segment of

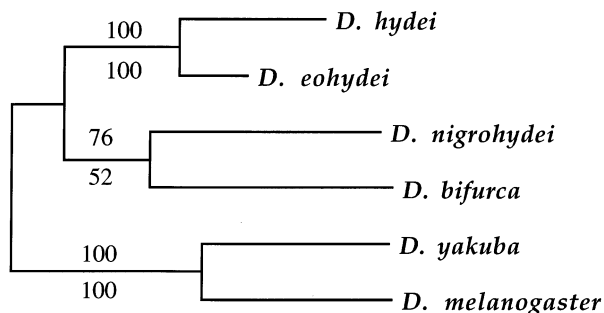


Fig. 2. Phylogeny of the *D. hydei* subgroup as inferred by unweighted parsimony and by transversal parsimony. The bootstrap values above the nodes indicate support for the unweighted parsimony analysis, while the values below the nodes show support for the transversal analysis. Both bootstrap analyses were accomplished by performing 300 repetitions. Branch lengths represent the amount of change for the unweighted parsimony analysis.

subunit I (*D. yakuba* positions 1783–2190), the entire subunit II (*D. yakuba* positions 3083–3766) comprising 688 bp, and a 419-bp segment of subunit III (*D. yakuba* positions 5015–5433). The *D. yakuba* positions refer to the Clary and Wolstenholme (1985) sequence. This results in a total of 1,515 bp sequenced for all four species.

Of the 1,515 nucleotide sites examined, the *D. hydei* subgroup taxa have 256 (17%) variable and 71 (5%) phylogenetically informative positions. However, there are 361 (24%) variable and 188 (12%) phylogenetically informative positions when the comparison includes the outgroup taxa *D. yakuba* and *D. melanogaster*.

The base composition bias statistics reveal that as reported for other insect protein-coding mtDNA genes the AT content is extreme (Clary and Wolstenholme 1985; DeSalle et al. 1987; Simon et al. 1994). Base composition bias is calculated according to Irwin et al. (1991) and ranges in value from between 0 to 1; 0 indicating no bias, and 1 showing complete base composition bias. A bias of 0.259 is calculated when considering only the ingroup taxa, but it is 0.268 when the outgroup is added for the comparison. The bias is even more severe when only the variable positions are considered, which is important because many nucleotide positions are not free to vary due to the constraint of having to maintain a conserved amino acid sequence. The variable position bias for the *D. hydei* subgroup is 0.339, but it becomes 0.395 when the outgroup taxa *D. yakuba* and *D. melanogaster* are included in the calculation.

The branch-and-bound unweighted parsimony analysis resulted in one most parsimonious tree, which is presented in Fig. 2. This tree has a length of 510 and a consistency index of 0.82 and a retention index of 0.60. The bootstrap values for the unweighted analysis all show significant values at the nodes, using the 70% or greater criterion of Hillis and Bull (1993). The same tree is produced when transversion parsimony is used, with a transversal tree length of 227. However, the bootstrap is not significant at the node defining the *D. bifurca*

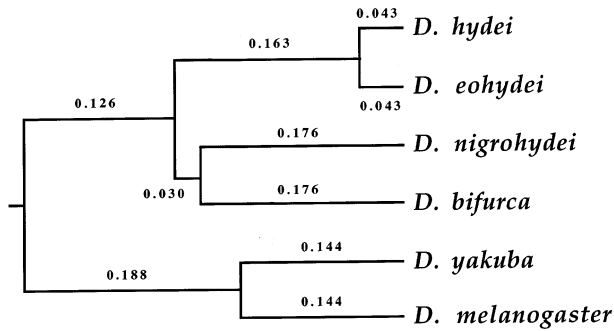


Fig. 3. The molecular clock phylogeny produced by the maximum likelihood analysis. As a reference point for absolute divergence times, Lachaise et al. (1988) place the divergence of *D. yakuba* and *D. melanogaster* at between 6 and 15 million years ago.

complex when only transversional changes are considered. When the *bifurca* complex is not considered to be monophyletic, the additional changes needed to be inferred are not very great. For example, if *D. nigrohydei* is included as a member of the *D. hydei* complex, the tree increases nine steps for a length of 519, and the transversional length increases by three steps to 230; conversely, if *D. bifurca* is considered as a member of the *D. hydei* complex, the unweighted parsimony length increases by four steps to 514, but the transversional parsimony length increases only one step to a length of 228.

The genetic distance estimates infer the same phylogenetic tree that the parsimony analyses produced. These include the *p*-distance, Jukes-Cantor, Kimura two-parameter, Tamura-Nei, Log Det/Paralinear, and the gamma distances clustered with either UPGMA or neighbor-joining. Bootstrap values for the genetic distance estimates are of the same magnitude as reported for the parsimony analyses; the *D. hydei* complex is highly supported, but only moderate to little support for the *D. bifurca* complex is observed.

A maximum likelihood analysis was performed by using the two-parameter model for unequal base frequencies of Hasegawa et al. (1985), with a discrete approximation to the Γ -distribution, and with the transition/transversion ratio and the shape parameter estimated according to the model. A total of ten rate categories were used (Yang 1994), and the average rate for each category was represented by the mean. Since the total base composition does not reflect the base composition of the positions that are free to vary (Spicer 1995), the base composition used in the maximum likelihood calculations was based only on the base frequencies of the variable positions (A = 0.344, C = 0.156, G = 0.047, T = 0.453). Parameter estimates from the maximum likelihood analysis produce an estimate of the transition/transversion ratio of 6.76 and an α value of 0.118. Since the maximum likelihood estimate should give the best branch-length estimates, this was used to create a tree that assumes rate constancy (Fig. 3). However, it should be noted that the molecular clock hypothesis was re-

jected based on the likelihood ratio test ($\chi^2 = 9.969$, $df = 4$, $P = 0.041$) of Felsenstein (1993).

Discussion

The results of these analyses seem to indicate that the taxonomic division of the subgroup into two complexes is warranted, since both appear to be monophyletic clades. This is very clear for the *D. hydei* complex, which is strongly supported by the molecular data set. However, this is not an unexpected finding given that this group was initially proposed based on what appear to be derived characters (see Fig. 1; specialized spermathecae and inversion 2z). But the monophyly of the *D. bifurca* complex, while much less definitive, is not anticipated by any character evidence, since all the characters that are currently used to define the complex appear to be plesiomorphic in nature. The uncertainty for the monophyly of the *D. bifurca* complex is due to the weak support it receives from the transversion analysis bootstrap. While the analyses which consider all nucleotide changes give the complex substantial support, the analyses which utilize only the transversional changes give very weak support. Since the taxa in question appear to be distantly related, more significance should probably be placed on the transversional analyses (Brown et al. 1982; Simon et al. 1994). Even though it seems that both complexes can be considered monophyletic, it should be noted that not all the taxa included in the subgroup were examined in our study. Although *D. neohydei* was not included, other evidence indicates that it is a member of the *D. hydei* complex. The inclusion of *D. novemariata* and *D. guayllabambae* in the *D. bifurca* complex is much less certain given that no currently known synapomorphic characters define either the complex or the within-species relationships. The examination of these species would be necessary for a more definitive statement.

The timing of speciation events in the *D. hydei* subgroup is difficult to establish. Unfortunately, no fossil representatives of the *D. hydei* subgroup, or even the *D. repleta* species group, have yet been found (Grimaldi 1987), and biogeographic reconstructions for the subgroup are of no assistance in determining absolute times of divergence. As a reference point for relative divergence times, Lachaise et al. (1988) suggest that the divergence of *D. yakuba* and *D. melanogaster* occurred sometime between 6 and 15 million years ago (mya), based on biogeographic considerations. Using the *D. yakuba*–*D. melanogaster* divergence times would infer that the species within the *D. hydei* subgroup diverged sometime between 9 and 21 mya. As for other relative divergence estimates based on molecular studies, only the divergence of the *D. repleta* species group, and of the *D. hydei* subgroup from some other *D. repleta* subgroups, can be evaluated. Beverley and Wilson (1984)

suggested a divergence of the *D. repleta* species group from some other subgenus *Drosophila* groups at about 35 mya based on immunological distance data. Roughly the same estimates were inferred by the two-dimensional electrophoretic data set of Spicer (1988), with the divergence of the *D. repleta* group occurring at about 32 mya and the divergence of the *D. mercatorum* subgroup from the *D. hydei* subgroup at about 22 mya. Finally, a combined analysis of all the alcohol dehydrogenase (ADH) gene sequences by Russo et al. (1995) suggests that the *D. hydei* subgroup diverged from the *D. mulleri* subgroup at about 14–15 mya. None of these estimates are incompatible with one another, but until more definitive absolute divergence times for some *Drosophila* lineages are established and more comparative sequence data are gathered it will be difficult to evaluate the reliability of molecular estimates.

These results are also interesting from the chromosomal evolution perspective. As mentioned previously, only one interspecific inversion (2z) has been noted for this subgroup, although several intraspecific variations are known (Wasserman 1982, 1992). Wasserman (1992) offered two alternative explanations for this observation: One is that the *D. hydei* subgroup is a recent assemblage of species, and the other is that it is an ancient group which has not undergone very much chromosomal change. Our results indicate that the latter hypothesis is correct, which was the alternative preferred by Wasserman (1992). As already mentioned, a great deal of evolutionary change has occurred with the sex chromosomes, but this alteration is unrelated to the chromosomal inversion evolution.

When examined in a phylogenetic context it appears that the interspecific crossability data are compatible with our inferred relationships. Within the *D. hydei* complex, only *D. hydei* and *D. neohydei* can produce interspecific hybrids. Fertile F₁ interspecific hybrids can be obtained by crossing either *D. hydei* males to *D. neohydei* females or *D. hydei* females to *D. neohydei* males (Wasserman 1962; Schäfer 1978). Although we did not examine *D. neohydei* in our study, the strong support that we show for the *D. hydei* complex, plus the ease of hybridization between *D. neohydei* and *D. hydei*, should be interpreted as suggesting close relationships among these species. As for the *D. bifurca* complex, only *D. bifurca* and *D. nigrohydei* have been tested, and they do not produce interspecific hybrids (Wharton 1944; Wasserman 1962). Interestingly, one intercomplex hybridization, between *D. eohydei* females and *D. nigrohydei* males, is successful. According to Wharton (1944) only two offspring were produced in 40 mass matings, but Wasserman (1962) found that in 80 pair matings not only could hybrids be produced, but that even some F₂ flies were noted. From a parsimonious perspective these results can be interpreted in two ways: The ability to hybridize was lost and then regained, or the ability to

hybridize was maintained and then independently lost. Given the current phylogeny, these two reconstructions cannot be differentiated, even though they potentially suggest different evolutionary processes. Without the specific genetic knowledge concerning the likelihood of gaining or losing the ability to hybridize, the only possible way to resolve this ambiguity would be to examine the other taxa in the subgroup in a phylogenetic context, but even this would not guarantee resolution.

Division of the subgroup into two complexes has interesting implications for consideration of the evolution of unusually long sperm in these species. Both *D. bifurca* and *D. hydei* have tremendously long sperm: 58.29 ± 0.66 mm and 23.32 ± 0.51 mm, respectively (Pitnick et al. 1955b; Pitnick and Markow 1994). Although the remaining two species examined in this study, *D. nigrohydei* and *D. eohydei*, also have long sperm: 15.00 ± 0.02 mm and 18.11 ± 0.27 mm, respectively (S. Pitnick, unpublished data; Pitnick 1996), they are considerably shorter than in the former two species. The phylogenetic affinities established here reveal that a very long and a much shorter sperm are found in each lineage, suggesting that sperm length is even more evolutionarily labile than previously believed. Examination of the remaining species of this subgroup will be required to fully discern the pattern of sperm length evolution among these species.

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